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Molecular Weights and Metabolism of Rat Brain Proteins

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1. Rats were injected with [U-^{14}C]glucose and after various intervals extracts of whole brain proteins (and in some cases proteins from liver, blood and heart) were prepared by high-speed centrifugation of homogenates in 0.9% sodium chloride or 0.5% sodium deoxycholate. 2. The extracts were subjected to gel filtration on columns of Sephadex G-200 equilibrated with 0.9% sodium chloride or 0.5% sodium deoxycholate. 3. Extracts prepared with both solvents displayed on gel filtration a continuous range of proteins of approximate molecular weights ranging from less than 2×10^4 to more than 8×10^5. 4. The relative amount of the large proteins (mol.wt. $>8\times10^5$) was conspicuously higher in brain and liver than in blood. 5. At 15min after the injection of [U-^{14}C]glucose the smaller protein molecules (mol.wt. $<2\times10^4$) were significantly radioactive, whereas no ^{14}C could be detected in the larger (mol.wt. $>2\times10^4$) protein molecules. The labelling of all protein samples was similar within 4h after injection of [U-^{14}C]glucose. Fractionation of brain proteins into distinctly different groups by the methods used in the present work yielded protein samples with a specific radioactivity comparable with that of total brain protein. 6. No evidence could be obtained by the methods used in the present and previous work to indicate the presence of a significant amount of 'metabolically inert protein' in the brain. 7. It is concluded that: (*a*) most or all of the brain proteins are in a dynamic state of equilibrium between continuous catabolism and anabolism; (*b*) the continuous conversion of glucose into protein is an important part of the maintenance of this equilibrium and of the homoeostasis of brain proteins *in vivo*.

The brain has been shown to contain a great variety of proteins, including lipoproteins and glycoproteins, which have been separated by using electrophoresis or DEAE-cellulose chromatography (Palladin, Belik, Polyakova & Silich, 1957; Moore & McGregor, 1965; Bogoch, 1968). However, little information appears to be available at present as to the range of molecular weights of brain proteins and the possible interrelationship between the molecular weights and metabolism of the various classes of proteins. In the present work gel filtration was used to obtain information relevant to this problem. This method is limited by the fact that the elution volumes of proteins from Sephadex columns are sensitive to the molecular shape of protein molecules as well as to their weight, and the results may also be influenced by dissociation of protein molecules as the result of dilution. However, for comparative purposes useful estimates of the molecular weight of proteins can be obtained by this method (Wieland, Duesberg & Determan, 1963; Determan, 1964; Andrews, 1964; Ackers, 1967).

In previous experiments we have observed that gel filtration of high-speed supernatants from rat brain homogenates results in separation of the brain cell-sap proteins into a continuous range of proteins of different molecular weights. These proteins were similarly labelled after the experimental animals were injected with [U-^{14}C]glucose 4h before decapitation (Vrba & Cannon, 1968). However, the high-speed supernatant contains only a part of the total tissue proteins. In experiments reported below we extracted an additional quantity of protein from the brain particulate matter by rehomogenization of this matter in 0.5% sodium deoxycholate. It is shown that gel filtration of the latter proteins is possible with the use of Sephadex G-200 columns, equilibrated with 0.5% sodium deoxycholate solutions.

Information on the metabolism of various groups of proteins separated by gel filtration was sought by prime labelling of the proteins *in vivo* with subcutaneous injections of [U-^{14}C]glucose. It has been shown that within less than 15min after the injection of labelled glucose more than 50% of the

^{14}C retained in the rat brain is incorporated into α-amino acids, and that this process is followed by an efficient incorporation of [^{14}C]glucose carbon into brain proteins within the next 5h (Vrba, 1962). This process is substantially more active in brain than in liver, kidney, heart, lungs, skeletal muscle, spleen or blood (Vrba, Gaitonde & Richter, 1962). Therefore this process appears to be particularly well suited for comparative observations of the degree of metabolic activity (or inertness) of different groups of brain proteins separated by gel filtration. Preliminary communications of some aspects of these experiments have been published (Vrba & Cannon, 1968, 1970).

METHODS

Animals. Brains of groups of six (or alternatively four) rats of a Wistar strain (weighing about 60g unless otherwise stated) were used in these experiments. The rats were conventionally maintained and fed with a Purina Rat Chow diet (≮22% crude protein, ≮4% crude fat, <5% crude fibre, <5% crude ash, according to the specification of the suppliers).

Materials. Sephadex G-200, laboratory columns (K 50/100, size 5cm×100cm) and Blue Dextran 2000 were from Pharmacia AB, Uppsala, Sweden. D(+)-[U-^{14}C]Glucose (2.8–2.9mCi/mmol, batches 141, 144, 145) and *n*-[1-^{14}C]hexadecane were supplied by The Radiochemical Centre, Amersham, Bucks., U.K.; crystalline albumin (bovine) and sodium deoxycholate were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; D(+)-glucose (anhydrous) was from British Drug Houses (Canada) Ltd., Toronto, Canada; sodium azide (purified) was from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.; non-enzymic protein molecular-weight markers for molecular-weight determination were from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; glucose oxidase blood-sugar kit was from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany; three-way Hamilton valves were from Hamilton Co., Whittier, Calif., U.S.A.

Preparation of extracts of brain proteins. After decapitation of the rats the brains (average wt. 1.2–1.3g) were excised and immediately frozen in liquid N_2. Six (in some experiments four) brains were collected, weighed to the nearest 0.01g and homogenized in about 50ml of precooled 0.9% NaCl for 5min at 500rev/min in a Potter–Elvehjem glass homogenizer with a Teflon pestle. The temperature during the homogenization was kept within the limits of 0–2°C (this temperature was maintained until the deproteinization of various protein fractions with $HClO_4$). The homogenate was made up to 60ml with 0.9% NaCl. After samples were taken for determination of protein and for preparation of the purified fractions, a 50ml portion of the remaining homogenate was centrifuged at 38000rev./min for 2h in a Spinco model L ultracentrifuge (no. 40 rotor) and the supernatant separated for further study.

In some experiments the sedimented particulate matter was resuspended in the original volume of 0.9% NaCl and homogenized again as described above. Homogenization followed by high-speed centrifugation was repeated consecutively three times in 0.9% NaCl and the resultant supernatants designated 'first, second and third NaCl supernatant' respectively. The sediment of particulate matter obtained after the third homogenization in 0.9% NaCl was then subjected to three homogenizations and centrifugations in 0.5% sodium deoxycholate. The resulting high-speed supernatants were designated 'first, second and third deoxycholate supernatant'. The residue was treated with 15ml of 6% (w/v) $HClO_4$ to separate non-protein substances. The protein was then subjected to the purification procedure given below and the material obtained was termed 'residual protein'.

Determination of protein, preparation of purified protein samples and measurement of radioactivity. From the various homogenates and supernatants portions were taken in triplicate and dissolved in an equal volume of 1M-NaOH. The volume was made up to 5 or 15ml with 0.5M-NaOH and after incubation overnight at 37°C protein was determined in triplicate by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine albumin dissolved in 0.5M-NaOH was taken through the analytical procedure together with the samples and used for preparation of triplicate standard curves. Precautions recommended by Hall & Cocking (1965) for protein determination by this method were strictly adhered to. When protein was determined in effluents from gel columns, the standards were prepared in solutions identical with the effluent.

From other portions of the brain dispersions or of the high-speed supernatants (usually 1, 5 or 15ml, according to the amount of protein present in the sample) purified protein samples were prepared for measurements of specific radioactivity. The purification of these samples consisted of: (*a*) precipitation of the protein with $HClO_4$ (final concn., 6%, w/v); (*b*) centrifugal washing (four times) of the precipitated proteins in large volumes of 6% $HClO_4$; (*c*) extraction of the acid-soluble sediment with ethanol–acetone (1:1, v/v) followed by extraction with chloroform–methanol–diethyl ether (2:1:1, by vol.) and ethanol–diethyl ether (3:1, v/v); (*d*) two extractions of the residue with hot 5% (w/v) trichloroacetic acid (90°C, 15min) to remove glycogen, nucleic acids and any residual free amino acids adsorbed on the residue (Vrba *et al.* 1962); (*e*) repeated extraction of the undissolved residue with two different organic solvents [diethyl ether–ethanol, 5:2 (v/v), followed by diethyl ether] to remove water and trichloroacetic acid; (*f*) drying of the residue in air. The latter was then dissolved in 0.5M-NaOH (final vol. 2–15ml). In suitable portions of this solution the amount of protein was determined in triplicate as described above; ^{14}C radioactivity was measured in other portions in triplicate in a Nuclear–Chicago model 720 liquid-scintillation counter. For calculation of radioactivity measurements the two-channels-ratio method was used and for a further control the radioactivity of each sample was counted again in the presence of an internal standard (*n*-[1-^{14}C]-hexadecane). The results of the radioactivity measurements are expressed as d.p.m. Further details of the preparative and analytical procedures employed were described by Vrba (1967).

Preparation of gel columns and examination of column effluents. Samples of Sephadex G-200 were suspended in about 100vol. (v/w) of 0.9% NaCl, or in 0.5% sodium deoxycholate. The particles were allowed to swell for

several days in the cold-room (0–2°C) and the smaller particles were removed by sedimentation and decantation ten times. After decantation under reduced pressure at 40°C the slurry was cooled in the cold-room, and two columns were prepared as described by Andrews (1964). The bed volumes were about 1700ml. The upward flow of the respective solvents was maintained for several days at a rate of approx. 20ml/h. Samples of protein extracts (20–50ml) or of molecular-weight markers were applied to the bottom of the column by using a syringe and a three-way surgical valve. Blue Dextran (10mg) and glucose (10mg) were added to each sample in the form of a 2ml solution to serve as markers of the void volume and of the elution volume of small-molecular-weight substances. The latter indicated the complete elution of the sample from the column. The recovery of added glucose was checked several times and found to be quantitative. The two columns were set up in a cold-room (0–2°C) and the flow of each solvent was kept constant even when the columns were not in use for protein separation. Under these conditions the repeated calibrations of the columns with molecular-weight markers were in satisfactory agreement. Cube roots of the distribution coefficient ($K_{av.}$) were calculated and plotted against the square roots of molecular weight (Porath, 1963; Determan, 1964; Wieland *et al.* 1963). Portions of the effluent (5ml each) were collected with an automatic fraction collector. Measurements of protein and ^{14}C radioactivity were made in suitable portions of each fraction as described above. Blue Dextran was determined by measuring the E_{625} and glucose by using glucose oxidase with the Boehringer Glucostat kit.

Preparative isolation of proteins from column effluents. Complete elution of the proteins applied to the column (including the added glucose) was achieved by collecting 400 fractions. Where indicated in the text, suitable batches of fractions were pooled, proteins were precipitated by addition of 0.1vol. of 60% (w/v) $HClO_4$ and sedimented by centrifugation. The sedimented proteins were then purified and their specific radioactivities measured as outlined above.

RESULTS

Calibration of columns. The columns of Sephadex G-200 were calibrated with protein molecular-weight markers. The results (Figs. 1 and 2) demonstrate that protein solutions in either 0.9% sodium chloride or 0.5% sodium deoxycholate can be separated by gel filtration on columns equilibrated with the same solvent. However, the separation of the marker proteins on the columns is influenced by the constituents of the eluting mixture, as the slopes obtained in Figs. 1 and 2 with sodium chloride and deoxycholate were different. Therefore it is important to calibrate such columns with markers dissolved in the solvent mixture identical with that to be used in separating proteins from tissue extracts if an assessment of their molecular weights is to be made.

Gel filtration of saline extracts of brain. The recovery of ^{14}C from the columns is illustrated in Fig. 3. Six young rats were injected subcutaneously with [U-^{14}C]glucose; 22h later the brains were excised and homogenized in 0.9% sodium chloride and a portion of the high-speed supernatant was subjected to gel filtration as described in the Methods section. The recovery of radioactivity expressed as the sum of ^{14}C in the 400 fractions collected represented 99% of the radioactivity of the sample before gel filtration.

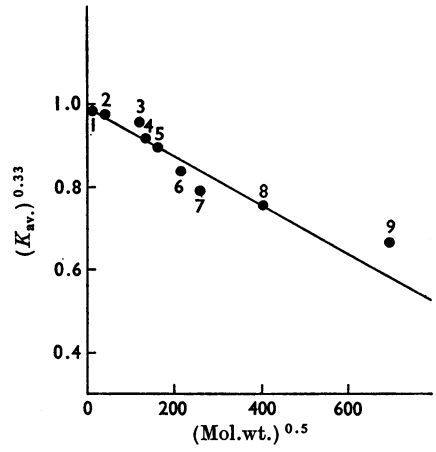

Fig. 1. Calibration of a column of Sephadex G-200 with molecular-weight markers. The bed volume was 1750ml and the volume of sample was 20ml. The eluent was 0.9% NaCl. The reference substances (their molecular weights and amounts in parentheses) were: 1, glucose (1.8×10^2; 23.2mg); 2, bacitracin (1.45×10^3; 6.4mg); 3, cytochrome *c* (horse heart) (1.24×10^4; 8.4mg); 4, myoglobin (cryst., sperm whale, salt-free) (1.78×10^4; 9.2mg); 5, chymotrypsinogen A (6× cryst., ox pancreas, salt-free) (2.5×10^4; 9.2mg); 6, ovalbuman (2× cryst.) (4.5×10^4; 9.8mg); 7, albumin (cryst., bovine) (6.7×10^4; 8.0mg); 8, γ-globulins (human) (1.6×10^5; 9.0mg); 9, apoferritin (amorphous, horse, salt-free) (4.8×10^5; 8.9mg).

The extract contains a continuous range of proteins with molecular weights ranging from less than 2×10^4 up to more than 8×10^5 (approx. molecular weight of proteins larger than the exclusion limits of Sephadex G-200). The high peak of the radioactivity that accompanied the elution of unlabelled glucose added to the sample as marker (Fig. 3, arrow no. 10) represents the low-molecular-weight radioactive components of the brain high-speed supernatant. Although the separation of proteins from the low-molecular-weight radioactive components was not quite perfect, it may be reasonably assumed that proteins having elution volumes (V_e) smaller than myoglobin (Fig. 3, arrow no. 7) are well separated from the low-molecular-weight components. However, the specific radioactivity of proteins having a molecular weight less than approx. 2×10^4 was measured by precipitation of these proteins with perchloric acid and

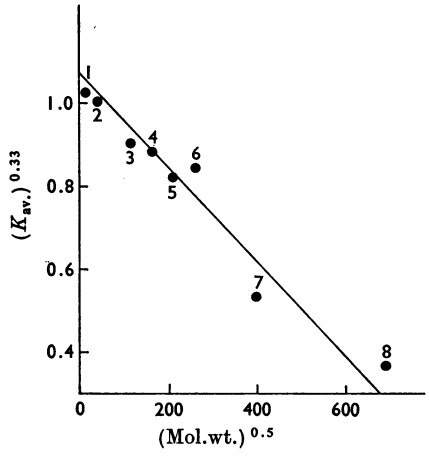

Fig. 2. Calibration of a column (K50/100) of Sephadex G-200 with molecular-weight markers. The bed volume was 1700ml and the volume of sample was 20ml. The eluent was 0.5% sodium deoxycholate. The reference substances (their molecular weights and amounts in parentheses) were 1, glucose (1.8×10^2; 20mg); 2, bacitracin (1.45×10^3; 8mg); 3, cytochrome *c* (horse heart) (1.24×10^4; 10.8mg); 4, chymotrypsinogen A (6× cryst., ox pancreas, salt-free) (2.5×10^4; 10.2mg); 5, ovalbuman (2× cryst.) (4.5×10^4; 9.5mg); 6, albumin (cryst., bovine) (6.7×10^4; 11.0mg); 7, γ-globulins (human) (1.6×10^5; 9.2mg); 8, apoferritin (amorphous, horse, salt-free) (4.8×10^5; 9.2mg).

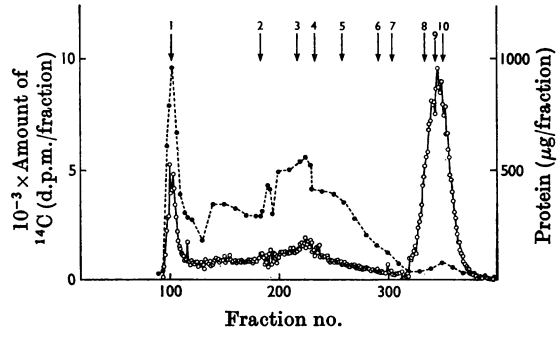

Fig. 3. Protein and ^{14}C content of fractions of high-speed supernatant of rat brain obtained 22h after injection of [U-^{14}C]glucose and separated by gel filtration on a Sephadex G-200 column. Brains from 6 rats (60g each) were pooled (wet wt. of brains 7.48g) and homogenized in 0.9% NaCl (final volume 50ml). The dose of [U-^{14}C]-glucose/animal was 1.369×10^8 d.p.m. The bed volume was 1750ml and the sample volume was 20ml. The eluent was 0.9% NaCl. The sample of brain high-speed supernatant applied to the column contained 4.76×10^5 d.p.m. Recovery of radioactivity (sum of all 400 fractions collected) was 99% (4.71×10^5 d.p.m.). Arrows at the top of the graph indicate the approximate molecular weights based on calibration of the column with markers as indicated in Fig. 1. 1, Blue Dextran (>8×10^5); 2, apoferritin (4.8×10^5); 3, γ-globulins (human) (1.6×10^5); 4, albumin (bovine) (6.7×10^4); 5, ovalbuman (4.5×10^4); 6, chymotrypsinogen (2.5×10^4); 7, myoglobin (1.78×10^4); 8, cytochrome *c* (1.24×10^4); 9, bacitracin (1.45×10^3); 10, glucose (1.8×10^2). ●, Protein; ○, ^{14}C.

elimination of radioactive contaminants by the purification of the proteins, as described in the Methods section.

Fig. 4 shows similar results obtained from brain extracts prepared at different times after injection of [U-^{14}C]glucose; 24 rats were injected with [U-^{14}C]glucose and groups of six animals were decapitated at intervals of 4, 8, 12 and 92h. The six brains of each group were pooled and homogenized in 0.9% sodium chloride and the high-speed supernatants were subjected to gel filtration. The high content of ^{14}C in the low-molecular-weight fraction had disappeared almost completely by 92h. The elimination of ^{14}C from the high-molecular-weight fractions (mol.wt.>2×10^4) is a much slower process.

The results of the experiments recorded in Fig. 4 do not allow us to conclude unequivocally that the ^{14}C present in the high-molecular-weight fractions eluted from the gel column occurs in the effluents in the form of protein carbon. To exclude the possibility that other high-molecular-weight components in the extracts may be the carriers of radioactivity, samples (equivalent to 90% of the effluent) were pooled to give four batches of effluents containing groups of proteins of different ranges of molecular weights, as indicated in Table 1. The pooled fractions were then subjected to the purification procedure described in the Methods section. Proteins of all ranges of molecular weights examined in the high-speed supernatants from homogenate of rat brain were labelled as soon as 4h after injection of [U-^{14}C]glucose (Table 1). However, it appears that the proteins with a smaller molecular-weight range were labelled first (Table 1, row *d*) and these also are the first to show a large loss of ^{14}C. Between 3.5 and 8h after injection of [U-^{14}C]glucose the decrease of specific radioactivity was highest in this fraction. Therefore an experiment was performed in which the animals were killed 15min after injection of labelled glucose.

The results showed that 15min after injection the high-molecular-weight proteins of the extract were free of any radioactivity; however, some ^{14}C was incorporated into the proteins of low molecular weights (Fig. 5). In this experiment the isolation and purification of the group of proteins of molecular weight below 2×10^4 yielded a protein preparation with a radioactivity of 930d.p.m./mg of protein.

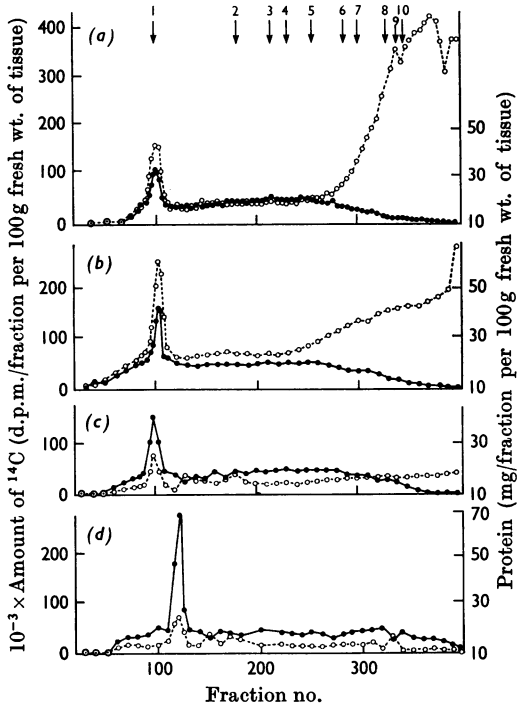

Fig. 4. Protein and ^{14}C content of fractions of high-speed supernatant of rat brain obtained at various times after injection of [U-^{14}C]glucose and separated by gel filtration on a column of Sephadex G-200. In each case a sample of brain high-speed supernatant obtained from six pooled brains was applied to the column. The injected dose of [U-^{14}C]glucose was 1.252×10^{8} d.p.m./animal. The bed volume was 1750ml and the sample volume was 20ml. The eluent was 0.9% NaCl. The time of decapitation of animals after injection of [U-^{14}C]glucose and amount of protein in the sample applied to the column were as follows: (*a*) 4h (113mg); (*b*) 8h (101mg); (*c*) 12h (106mg); (*d*) 92h (111mg). Arrows on the top of the graph indicate the approximate molecular weights as indicated in Fig. 3. ●, Protein; ○, ^{14}C radioactivity.

In the purified protein samples prepared from the fractions of molecular weight higher than 2×10^{4}, no measurable amounts of ^{14}C were found. These observations confirm some previous experiments that demonstrated the occurrence of ^{14}C in rat brain proteins as soon as 5–15min after injection of [U-^{14}C]glucose (Vrba, 1962; Elliott & Yoshino, 1968). The present results indicate that the earliest labelling of brain proteins with [^{14}C]glucose carbon is limited to the small protein molecules. However, 4h later the label was present in all high-speed supernatant proteins (Table 1).

Gel filtration of saline extracts of liver, heart and blood. Fig. 3 shows that a relatively large proportion of the total protein was recovered in the void

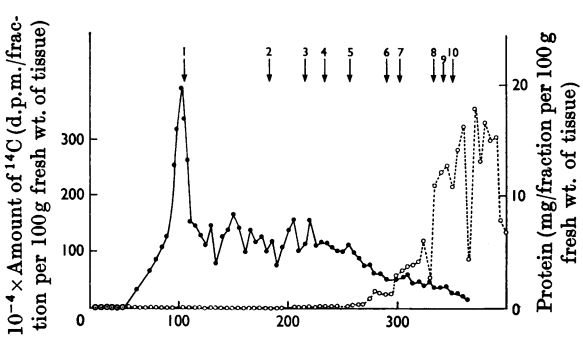

Fig. 5. Protein and ^{14}C content of fractions of high-speed supernatant of rat brain obtained 15min after injection of [U-^{14}C]glucose and separated by gel filtration on a column of Sephadex G-200. A sample of high-speed supernatant obtained from four pooled brains was applied to the column. The injected dose of [U-^{14}C]glucose/animal was 2.509×10^{8} d.p.m. The bed volume was 1750ml and the sample volume was 20ml (43mg of protein). The eluent was 0.9% NaCl. Arrows on the top of the graph indicate the approximate molecular weights as indicated in Fig. 3. ●, Protein; ○, ^{14}C radioactivity.

Table 1. *Specific radioactivities (d.p.m./mg of protein) of groups of proteins of different molecular weights in high-speed supernatants from rat brain homogenate obtained at various intervals after injection of* [U-^{14}C]*glucose*

In each case a sample of high-speed supernatant obtained from six pooled brain homogenates was applied to a Sephadex G-200 column. After gel filtration (see Fig. 4) suitable batches of eluted fractions were pooled and purified samples of proteins were prepared from each batch as described in the Methods section. The approximate range of molecular weights of proteins has been estimated from values of $K_{av.}$ (Fig. 1). For all other conditions see the legend to Fig. 4.

	Sp. radioactivity (d.p.m./mg of protein)			
Time between injection of [U-^{14}C]glucose and decapitation of rats ...	3.5h	8h	12h	92h
Approx. mol.wt. range of protein samples				
(*a*) $>8\times10^{5}$	3390	4100	1110	1070
(*b*) 8×10^{5}–5×10^{4}	3850	3800	1280	1150
(*c*) 5×10^{4}–2×10^{4}	3100	2460	1040	1080
(*d*) $<2\times10^{4}$	4560	2760	980	1150
(*e*) Protein sample of the high-speed supernatant before gel filtration (i.e. sample of total cell-sap protein)	3510	3960	1400	1150

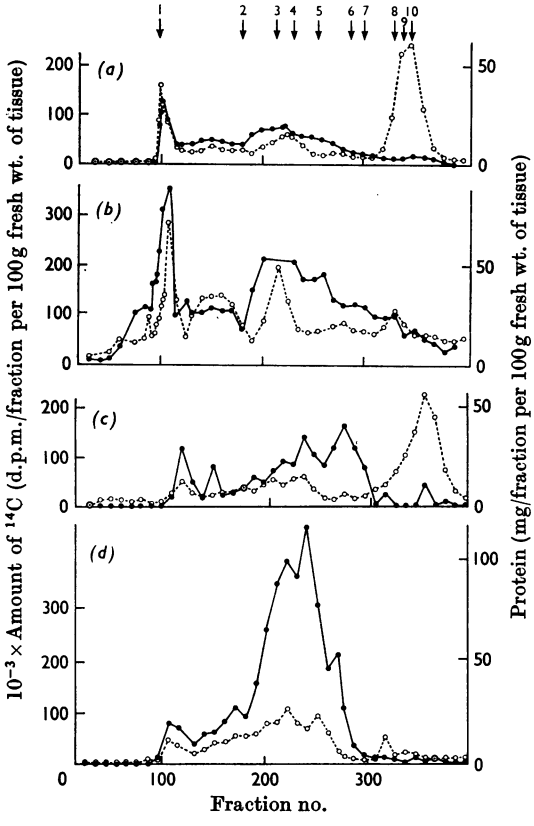

Fig. 6. Gel filtration on a column of Sephadex G-200 of brain, liver, heart and blood high-speed supernatants obtained 22h after injection of [U-^{14}C]glucose. In each case a sample of the high-speed supernatant, obtained from six pooled organs, was applied to the column. The injected dose of [U-^{14}C]glucose was 1.369×10^8 d.p.m./animal (60g). The wet weights of six pooled organs were: brain, 7.48g; liver, 11.10g; heart, 1.78g; blood, 4.00g. The final volume of homogenates was in each case 50ml except for liver (final volume 150ml). The bed volume was 1750ml and the sample volume was 20ml. The eluent was 0.9% NaCl. (*a*) Brain; (*b*) liver; (*c*) heart; (*d*) blood. Arrows on the top of the graph indicate the approximate molecular weights as indicated in Fig. 3. ●, Protein; ○, ^{14}C radioactivity.

volume. Similar extracts of brain, liver, heart and blood were therefore subjected to gel filtration for comparative purposes (Fig. 6). The soluble proteins of brain and liver contain a much higher proportion of large molecules (mol.wt. $> 8 \times 10^5$) than the blood. The heart occupies an intermediate position in this respect.

Gel filtration of deoxycholate extracts of brain. The results recorded in Figs. 3–6 relate only to 'cell-sap' proteins, present in the high-speed supernatants of the tissue homogenates prepared in 0.9% sodium chloride. However, the proteins remaining in the sediment are of considerable interest. The distribution of brain protein in consecutive brain protein extracts is shown in Table 2. The first extract in 0.9% sodium chloride contained the bulk of the cell-sap proteins, representing about 35% of the total protein recovered. Additional homogenizations in 0.9% sodium chloride extracted only small and rapidly decreasing quantities of protein. However, a considerable amount of protein was extracted by rehomogenizing the sediment in 0.5% sodium deoxycholate. Additional homogenization in sodium deoxycholate achieved only a small further extraction of proteins from the particulate matter. It is noteworthy that all seven protein fractions obtained in this way were labelled to a degree comparable with that of the total brain proteins within 4h after injection of [U-^{14}C]glucose.

Purified protein samples of different ranges of molecular weights were prepared from both types of high-speed supernatant as described in the Methods section. The results (Table 3) show that, although the specific radioactivity of various groups of proteins varied, the differences were not striking; nor did any subfraction differ in the specific radioactivity from the sample of the total brain proteins by more than ±30% for those prepared with sodium chloride or ±10% for those prepared with deoxycholate.

DISCUSSION

The present experiments appear to indicate that few, if any, of the rat brain proteins are metabolically inactive. Even though the constituent high-molecular-weight proteins of both the sodium chloride and deoxycholate extracts that emerged from the columns in the void volume were not separated on Sephadex G-200, it seems unlikely that they contain less metabolically active proteins. If that were the case, the specific radioactivity would have been lower than of the other separated proteins; this is not so, as shown by the results in Table 1 (row *a*) and Table 3. It seems unlikely therefore that further separation of these high-molecular-weight proteins would result in the detection of significant amounts of unlabelled (metabolically inert) protein.

Other similar experiments in which proteins of brain (labelled with [U-^{14}C]glucose *in vivo*) were fractionated by differential centrifugation, sucrose-density-gradient centrifugation or fractional precipitation with ammonium sulphate also yielded proteins that were labelled; no fraction with either extremely high or extremely low specific radioactivity (in comparison with that of the total rat brain proteins) could be obtained (Vrba *et al.* 1962; Vrba, 1967; Vrba & Sheehan, 1968; Vrba & Cannon,

Table 2. *Distribution and specific radioactivity of proteins obtained from high-speed supernatants of rat brain homogenates prepared by consecutive homogenizations of rat brains in 0.9% sodium chloride and 0.5% sodium deoxycholate.*

Six rats (90g each) were injected with 2.73×10^8 d.p.m. of [U-^{14}C]glucose and decapitated 4h later. A homogenate of six pooled brains in 0.9% NaCl was prepared and subjected to high-speed centrifugation. Consecutive high-speed supernatants were prepared as described in the text and determinations of the amount of protein and of specific radioactivity of a purified protein sample from each high-speed supernatant were carried out as described in the Methods section.

	Distribution of protein (% of recovered protein)	Sp. radioactivity (d.p.m./mg of protein)
All brain proteins*	—	1890
First NaCl supernatant	35.2	1560
Second NaCl supernatant	7.6	2170
Third NaCl supernatant	2.4	1790
First deoxycholate supernatant	32.7	1390
Second deoxycholate supernatant	5.7	1420
Third deoxycholate supernatant	2.3	1280
Residual protein	14.3	2780

*Proteins of the brain homogenate before high-speed centrifugation.

1970). The continuous incorporation of glucose carbon into the bulk of brain proteins indicates that most, if not all, of the brain proteins are turning over. The unchanging concentration of protein in the normal mature brain can be explained by a dynamic state of equilibrium between catabolism and anabolism of brain proteins. The continuous conversion of glucose into protein is a part of the maintenance of this equilibrium. This conclusion should also influence our views on the role of glucose in brain metabolism and function (Vrba & Cannon, 1970).

Our experiments are in good accordance with the results of Lajtha & Toth (1966) who also concluded that only a very small fraction, if any, of the cerebral proteins is metabolically stable. The results also indicate that the dynamic state of all of the investigated groups of brain proteins may be observed within hours rather than weeks if, instead of radioactive lysine as used by Lajtha & Toth (1966), labelled glucose is used as precursor for the carbon skeleton of the brain protein. Use of labelled glucose offers the advantage that the amino acids being incorporated into proteins are rapidly labelled *in situ* (Vrba, 1962) and therefore avoids dependence on passage of labelled amino acid through the blood–brain barrier. It is known that the passage of most amino acids through the blood–brain barrier is impeded, whereas this is not so for glucose (Dobbing, 1961).

Comparison of our results with those of others

Table 3. *Specific radioactivity of rat brain proteins in different extracts and of different molecular weights in fractions obtained by gel filtration of high-speed supernatants of rat brain homogenates*

First NaCl supernatant and first deoxycholate supernatant (Table 2) were subjected to gel filtration. Suitable batches of eluted fractions were pooled and purified samples of proteins were prepared from each batch as described in the Methods section. The approximate range of molecular weights of proteins was estimated from values of $K_{av.}$ of the elution volumes of the pooled fractions by using the calibration curves of the Sephadex G-200 columns (Figs. 1 and 2). (*a*) The bed volume was 1700ml and the volume of sample was 36ml (216mg of protein). The eluent was 0.9% NaCl. (*b*) The bed volume was 1700ml and the volume of sample was 50ml (280mg of protein). The eluent was 0.5% sodium deoxycholate.

	Approximate mol.wt. range of proteins separated by gel filtration	Sp. radioactivity (d.p.m./mg of protein)
Before gel filtration (total proteins)		1560
(*a*) First NaCl high-speed supernatant	$>6.3\times10^5$	1800
	6.3×10^5–2.6×10^5	1760
	2.6×10^5–1.2×10^5	1540
	1.2×10^5–4.6×10^4	1360
	4.6×10^4–1.2×10^4	1100
Before gel filtration (total proteins)		1390
(*b*) First deoxycholate high-speed supernatant	$>8\times10^5$	1370
	8×10^5–1.6×10^5	1340
	1.6×10^5–6.8×10^4	1450
	6.8×10^4–4.0×10^4	1530
	4.0×10^4–7.2×10^3	1310

suggests that in the intact animal the fate of ^{14}C from [U-^{14}C]glucose is quantitatively different from that observed *in vitro*. Thus *in vivo* there occurs a relatively rapid incorporation of ^{14}C from [U-^{14}C]-glucose into free amino acids, proteins and lipids, in both brain and other organs (Vrba, 1962, 1966; Vrba *et al.* 1962; Elliott & Yoshino, 1968). In contrast with these observations, in experiments *in vitro* a significantly larger proportion of ^{14}C from glucose appears in lactic acid and a significantly smaller proportion is incorporated into free amino acids and the acid-insoluble fraction of the tissue, which includes proteins and lipids (Chain, Larsson & Pocchiari, 1960). The possible physiological significance of the difference between the fates of labelled glucose carbon *in vivo* and *in vitro* has been discussed by Vrba & Cannon (1970).

The arterial–venous difference in oxygen, carbon dioxide, pyruvic acid and lactic acid in the brain *in situ* is in good agreement with the stoicheiometry required for complete dissimilation of glucose in brain (Himwich, 1951; McIlwain, 1966). However, the $^{14}CO_2$ in the venous blood of a cat brain perfused with a [^{14}C]glucose-containing perfusion fluid had a lower specific radioactivity than the labelled glucose in the 'arterial' blood supplied to the perfused brain (Allweiss & Magnes, 1958*a*,*b*; Geiger, 1958; Geiger, Kawakita & Barkulis, 1960; Gainer, Alweiss & Chaikoff, 1963). It seems reasonable to conclude that a part of the carbon dioxide in the brain venous blood is derived from catabolism of non-glucose substrates, whereas a part of the arterial blood glucose, which normally serves as the main, if not the only, source of glucose for the brain *in situ* is utilized there for reconstitution of the totally oxidized non-glucose substrates. The experiments recorded in the present paper demonstrate that: (*a*) glucose in the brain is continuously utilized for conversion into a rich variety of brain proteins; (*b*) the constant amount of protein in the brain can be explained only by a continuous utilization of the brain proteins; (*c*) the glucose–protein interrelationship represents an important part of the brain homoeostasis. Considering the relatively high rate of incorporation of glucose carbon into α-amino acids in the brain when compared with other organs (Vrba *et al.* 1962), the continuous conversion of glucose into protein in the brain may give a clue to the well-known fact that the brain is unusual among the larger organs in its dependence on glucose (Himwich, 1951; McIlwain, 1966). The process of utilization of non-glucose substrates (including proteins) in the brain, which is a counterpart of the constant assimilation of glucose in the maintenance of brain homoeostasis, has been discussed elsewhere (Vrba, 1955, 1956, 1957, 1962; Vrba, Folbergrová & Kantůrek, 1957; Vrba & Folbergrová, 1959, 1961).

Until the present time no significant brain arterial–venous difference of nitrogenous substances has been observed that could account for a continuous breakdown of brain proteins (Himwich, 1951; McIlwain, 1966). It has to be assumed that ammonia and other nitrogenous products of protein catabolism are retained in the brain and reutilized there. Cyclic production and reutilization of ammonia in nervous tissue was postulated 35 years ago (Krebs, 1935), but relatively little attention has been paid to this concept since. Experiments *in vivo* reported elsewhere appear to support the hypothesis of a cyclic formation and reutilization of nitrogenous substances in the brain in connexion with brain function (Vrba, 1955, 1956, 1957).

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